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一种新的用于提高*lea3*基因扩增特异性和产量的增强剂[*]

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摘要：在对目的DNA序列尤其是高GC含量的片段进行PCR扩增的时候，经常需要对一些试验条件进行优化。在一定条件下，二甲基亚砜、甲酰胺、甘油、NP-40和Tween20等可以在某种程度上提高PCR的特异性和效率。我们在对禾本科*lea3*基因进行分离克隆时发现了一种新的可以提高PCR产量和特异性的物质——极高热稳定单链结合蛋白（ET SSB），研究发现在每50 μl PCR反应体系中加入200 ng的ET SSB，可以有效地抑制DNA片段的非特异性条带的产生，并可以提高目的片段的产量。

关键词：高GC含量；聚合酶链式反应；极高热稳定单链结合蛋白
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Effect of New Specificity and Yield Enhancer on Amplification of *lea3* Gene

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Abstract：Amplification of a target DNA by the polymerase chain reaction (PCR) often requires experimental optimization factors especially when the target sequence contains high content of GC. Dimethyl sulfoxide，formamide，glycerol，NP-40 and Tween20 are known to increase，the specificity and efficiency of PCR under certain conditions. In the amplification of *lea3* gene in Gramineae，we found a new Extreme Thermostable Single-Stranded DNA Binding Protein (ET SSB) and improved yield of PCR. Addition of 200 ng of ET SSB per 50 μl reation decreased the formation of non-specific DNA fragments and increased the yield of specific PCR products.

Key words：High GC content；PCR；ET SSB

A number of PCR additives are now commercially available，however the identities of these agents are not usually revealed by their suppliers. Frackman *et al*. (1998) have demonstrated that Betaine is available at a fraction of the cost as a 5 mol·L^{-1} solution，and be sure to use Betaine or Betaine (mono) hydrate and not Betaine HCl. DMSO at 2%－10% may be necessary for amplification of some templates，however 10% DMSO can reduce *Taq* polymerase activity by up to 50% (Gelfand，1988). DMSO is thought to reduce secondary structure and is particularly useful for GC rich templates. Formamide is generally used at 1%－5% and 10% formamide is reported (Gelfand，1989) to have no effect on the activity of *Taq* polymerase，however，Sarkar *et al*. (1990) found that 1.25% formamide worked as well as 2.5% and 5%，and no

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作者简介：于丽霞（1981－）女，助教，主要从事植物分子生物学研究。E-mail：yulixia2005@gmail.com amplification was seen at 10% so it seems prudent not to use concentrations of formamide greater than strictly necessary for optimal amplification. Non-ionic detergents stabilize *Taq* polymerase and may also suppress the formation of secondary structure. 0.1%－1% Triton X-100, Tween 20 or NP-40 may increase yield but may also increase non-specific amplification (Gelfand, 1989). TMA is generally used at a final concentration of 2 mmol·L^{-1} to eliminate non-specific priming (Kovarova and Draber, 2000). BSA has proven particularly useful when attempting to amplify ancient DNA or templates which contain PCR inhibitors such as melanin. And recently, it was found that the PCR could be dramatically enhanced by Au nanoparticles (Li *et al.*, 2005).

Single-Stranded DNA Binding Protein (SSB) keeps the strands separate from tending to revert to the duplex form and allowing the DNA replication machinery to perform its function. Single-stranded DNA (ssDNA) binding proteins have been assigned the role of removing secondary structure in DNA and protecting ssDNA from hydrolysis by nucleases (Chase and Williams, 1986). However, in addition to these mundane roles, ssDNA binding proteins are now recognized as a key component of the replisome where they physically and functionally interact with other replication proteins and with the primer-template (Benkovic *et al.*, 2001; O′Donnell, 2006; Hamdan and Richardson, 2009). ssDNA binding-proteins are also engaged in DNA recombination and repair (Lohman and Ferrari, 1994). At present, SSB was also used for RNA targets tested (Goldmeyer *et al.*, 2007). In view of these multiple roles it hasn′t been reported that it can remarkably improve the amplification of high GC content sequences especially *lea3* gene. In this research, it was found that it was particularly useful for GC rich templates when 200ng of Extreme Thermostable Single-Stranded DNA Binding Protein (ET SSB) per 50 μl reaction was added in polymerase buffer, which indicated it could remarkably increase the yield and specificity of PCR reaction. Most of important, it was convenient and efficient that adding ET SSB in nucleic acid amplification comparing to other additives.

Group 3 LEA (Late embryogenesis abundant) proteins include 11 amino acids (TAQAAKEKAGE), resulting in amphypathic-helixed structure during dehydration of higher plants (Dure, 1993). Unlike most of eukaryotic genes which contain GC-rich sequences mainly in the promoter region, GC content of *lea3* gene is higher in all coding region.

Materials and Methods

Amplification reactions were carried out under the following conditions: 10 mmol·L^{-1} Tris-HCl pH 8.8, 50 mmol·L^{-1} KCl, 1.5 mmol·L^{-1} $MgCl_2$, 0.2 μmol·L^{-1} primers, 0.5 μg template, 0.2 mmol·L^{-1} each dNTP, 2.5 U Taq polymerase. The templates were cDNA and genomic DNA of barley. Total genomic DNA was isolated using DNA quick Plant System. Taq polymerase and dNTP were obtained from TransGen Biotech. Primers were synthesized by Shanghai Sangon Biological Engineering Technology & Service Company Ltd. The sequences of primers were as follows: forward, 5′-atggcctccaaccagaaccagg-3′, reverse, 5′-ctagtgattcctggtggtggtgg-3′. Additive reagents were obtained from the following sources. DMSO, formamide, glycerol and Tween20 were purchased from Amresco, NP-40 were purchased from Fluko. ET SSB was purchased from Biolabs.

The cycling conditions in a Biometer® T Gradient thermal cycle were an initial incubation for 5 min at 94℃ followed by 35 cycles each of 30s of denaturation at 94℃ and 30s of annealing at 62℃ for 50s of extension at 72℃, a final elongation step at 72℃ for 7 min. Amplified materials were subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide, visualized on a UVP Imaging System and documented by VisionWorksLS.

Recovery of amplified fragments was conducted under manufactory description of Gel Extraction Mini Kit (Huashun, Shanghai, China). The connection of T carrier from recovery adopts pMD18-T Vector Systems (TaKaRa, Dalian, China). By DNA transformation techniques, transformed susceptibility cells were cultured on 50 mg·L^{-1} ampicillin plate for 9－16 h under 37℃. After single clone amplification in LB, harvested product used for sequence analysis by Shanghai Shenggong Biotechnique Company, Lt.

Results and Discussion

Primers were designed according to the reported sequences of barley *lea3* gene (GenBank ID: FJ026804). Our preliminary experiments showed that it was very difficult to amplify *lea3* gene with genomic DNA of barley as template without any additive. In other previous research, DMSO or combination of betaine and DMSO was used for the amplification of sequences with high GC content (Kang *et al.*, 2005).

Unlike most house-keeping genes which contain CpG islands in their promoter region that are limited regions of high-density CpG residues, the GC content of HVA1 gene was high. However, we could amplify the target sequences without any additive when positive *Escherichia coli*. (DH5α) strain including HVA1 gene was used as template. Thus, we considered that the additive could improve the problem in our research because: 1. reduce secondary structure and is particularly useful for GC rich templates; 2. additives possibly decrease the Tm of GC rich sequences; 3. protect template DNA from apurinic/apyrumidinic effects of high temperature; 4. protect Taq DNA polymerase from high temperature.

In this paper, it was found that ET SSB could remarkably improve both the yields and the specificity of amplification of sequences with high GC content, while DMSO of concentration from 3% to 15% could just improve the yields. And the yield of PCR increased when the concentration of DMSO changed from 3% to 10%, which was similar to Bookstein′s, while the product could be tested even when the concentration of DMSO increased up to 15%. Only minor target products could be attained in the presence of low concentration (from 1% to 3%) of formamide. Unlike the reported that glycerol could improve the amplification of high (G＋C) templates, glycerol with the concentration from 1% to 5% was not helpful to amplify the fragments of *lea3* gene. Although non-ionic detergents such as 0.1%－1% Tween 20 or NP-40 are commonly used in sample processing protocols (Gelfand, 1988), these reagents had no contribution to amplification of *lea3* gene in our experiment. Among the combinations of DMSO and glycerol at different concentration, it was found that only combination of 1% glycerol and 5% DMSO could increase the yield of product slightly, others could not improve the amplification of *lea3* gene (Fig. 1).

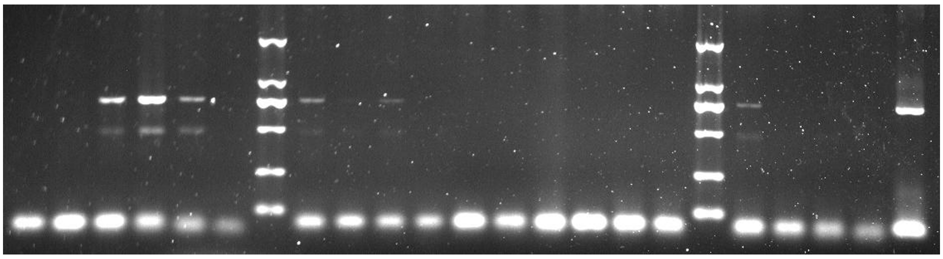

Fig. 1　The effects of different additives on PCR

M. DL2000 marker; Lane 1-21 None additive as control; 2% DMSO; 5% DMSO; 10% DMSO; 15% DMSO; 20% DMSO; 1% formamide; 2% formamide; 3% formamide; 1% glycerol; 3% glycerol; 5% glycerol; 0.2% Tween20; 0.8% Tween20; 0.2% NP-40; 0.8% NP-40; combination of 1% glycerol and 5% DMSO; combination of 2% glycerol and 5% DMSO; combination of 1% glycerol and 10% DMSO; combination of 2% glycerol and 10% DMSO; 4 ng·μl^{-1} ET SSB

Moreover, we have succeeded in attaining cDNA fragments of *lea3* gene with high GC content from 68% to 73% in several Gramineae plants including *Ampelocalamus calcareous*, *Sorghum vμlgare*, *Cynodon dactylon*, *Saccharum officinarum*, *Zizania latifoli* and *Oryza officinalis* (Fig. 2), with a certain concentration of ET SSB, from which we considered that ET SSB might be helpful to all sequences with high GC content.

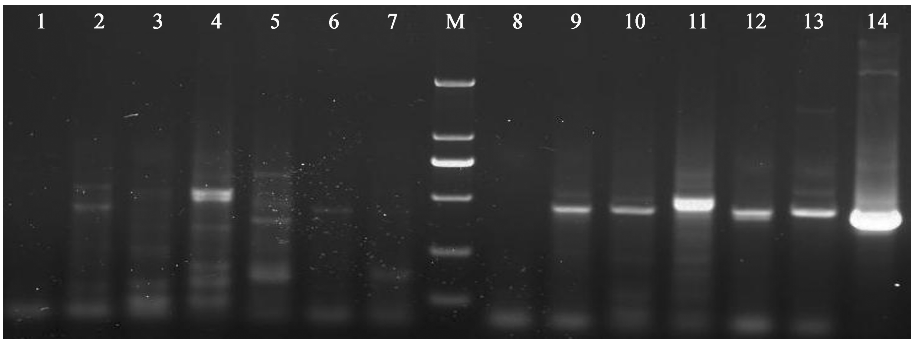

Fig. 2 cDNA fragments of *lea3* genes in different plants

M. DL2000 marker; Lane 1 blank with no additive; Lane 2-7 *Ampelocalamus calcareous*, *Sorghum vμlgare*, *Cynodon dactylon*, *Saccharum officinarum*, *Zizania latifoli* and *Oryza officinalis* with no additive; Lane 1 blank with ET-SSB; Lane 9-14 *Ampelocalamus calcareous*, *Sorghum vμlgare*, *Cynodon dactylon*, *Saccharum officinarum*, *Zizania latifoli* and *Oryza officinalis* with ET-SSB

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